APPENDIX C

An element-by-element comparison of Mehlhorn claims 27-50 with the Nichols Reference

Nichols is prior art under § 102(b) as of its November 11, 1976 publication date. As is evident from Claim Chart I, below, Nichols teaches steps (a) through (c) of the claimed method of independent claims 27 and 38 which call for preparation of a "liposome vesicle-entrapped charged chemical species."

In essence, step (a) of these claims calls for forming liposomes in an aqueous medium containing an acid in substep (i), or a base in substep (ii), "which is substantially impermeable through the vesicle." Mehlhorn has defined "substantially impermeable" as meaning that the degree of permeability of the acid or base is sufficiently low to maintain a pH gradient during loading.[1] The result of this step is a liposome containing an (i) acidic, or (ii) basic, aqueous

---

[1] Prosecution History of USSN 07/741,305, Paper, No. 9, Amendment, March 22, 1993, at p. 10 states (emphasis added):

> In the present case, applicant maintains that, in view of the specification, one skilled in the art would readily comprehend what is meant by the recitation of a "substantially impermeable" vesicle. More particularly, as discussed previously, the acid or base must not be able to permeate the vesicle membrane because it is essential to maintain a pH gradient between the inner and outer walls of the vesicle. Quite clearly, therefore, in reciting that the acid or base is substantially impermeable through the vesicle, applicant intends to convey that amounts of base or acid which do permeate the wall are not enough as to meaningfully interfere with the gradient which is the driving force for loading a charged species into the vesicles. Thus, a person skilled in the art would clearly know what is meant by the employed terminology and the rejection should therefore be withdrawn.

medium.[2] In Nichols, liposomes were prepared containing catecholamines, which are relatively lipophilic or hydrophobic drugs, by first forming liposomes containing citric acid, to which the liposomes were substantially impermeable, as evidenced by the formation of a pH gradient upon addition of a base to the external medium. (See Nichols Declaration, at ¶ 8, FR vol II, tab 6 at 5).

Step (b) of the two independent claims calls for adding (i) a cationic chemical species to the acidic liposome medium, or (ii) an anionic chemical species to the basic liposome medium. Nichols added the catecholamines dopamine, epinephrine, and norepinephrine. These catecholamines are cationic species which are relatively lipophilic, as shown by their ability to cross a lipid membrane. (See Nichols Declaration, at ¶ 11, FR vol II, tab 6 at 6; Prestegard Declaration, at ¶ 9, FR vol II, tab 8 at 15-16). Indeed, the involved Forssen patent describes dopamine and epinephrine as "cationic, lipophilic drugs which can partition into a lipid bilayer." Col. 3, lines 30-36. Accordingly, Nichols discloses the addition of cationic lipophilic drugs to an acidic liposome medium of substep (i) and, by analogy, the addition of an anionic chemical species to the basic medium of substep (ii).

Step (c) of claims 27 and 38 calls for adding a (i) base, or (ii) acid, to the (i) cationic, or (ii) anionic, species to induce it to pass into the liposome's internal (i) acidic, or (ii) basic phase, by means of a pH gradient (recited expressly in claim 38 but implicitly in claim 27). Nichols discloses addition of the base NaOH to create a gradient of 3 pH units causing the catecholamine

---

[2] Mehlhorn has admitted that the inducement of a cationic chemical species into an internal acidic aqueous solution of a liposome by a base is not "patentably distinct" from the inducement of an anionic species by addition of an acid. See Prosecution History of USSN 07/741,305, Paper No. 13, Response to Restriction Requirement, July 23, 1993, at page 2; Paper No. 16, Amendment and Renewed Request for Interference, April 22, 1994, at pp 3-4.

added in step (b) to accumulate inside the liposome by the mechanism described above. (See Nichols Declaration, at ¶ 9, FR vol II, tab 6 at 5). As noted above, it would have been obvious to analogously add a base to induce the anionic species into the liposome. Mehlhorn calls for (b) adding the cationic chemical species to be loaded, then (c) adding a base to create a pH gradient to load that species into the liposome. The Nichols model contains each of these steps, but the pH gradient of step (c) is created prior to adding the cationic drug to be loaded into the liposome of step (b). This change in sequence of steps is of no scientific significance. Dr. Prestegard explains that no unexpected result or advantage is observed by the obvious reversing of the order of these two steps. (See Prestegard Declaration, ¶ 13, FR vol II, tab 8 at 18). Dr. Nichols agrees. (See Nichols Declaration, at ¶ 13, FR vol II, tab 6 at 6-7).

Accordingly, Nichols teaches each element of the claimed method of steps (a), (b), and (c) of independent claims 27 and 38. The correlation between the subject matter claimed in Mehlhorn Claims 27-50 and the subject matter disclosed by Nichols is summarized in the Claim Chart I below:

Claim Chart I

Mehlhorn	Prior Art: Nichols or Nichols in view of Cramer
27. A method of preparing a liposome vesicle-entrapped charged chemical species which comprises:	Nichols teaches a method of preparing liposomes containing catecholamines [p. 269, Summary, lines 2-4], by:
(a) forming liposomes in: (i) an aqueous medium containing an acid	forming liposomes from egg phosphatidylcholine in an aqueous citrate-phosphate buffer containing citric acid [p. 270, lines 5-7]
which is substantially impermeable through the vesicle	which is "substantially impermeable" through the vesicle (as evidenced by establishment of a pH gradient upon addition of sodium hydroxide to the external liposome phase [p. 270, lines 10-12; March 22, 1993 Amendment, Paper No. 9, p. 10, lines 7-17])
to give an acidic liposome-containing aqueous medium in which the acid is present in the internal and external liposome phases; or	to give an aqueous citrate-phosphate buffer at pH 5 containing liposomes; citric acid is present in both the internal and external liposome phases [p. 270, lines 7-9];
(ii) an aqueous medium containing a base which is substantially impermeable through the vesicle to give a basic liposome-containing aqueous medium in which the base is present in the internal and external liposome phases;	Mehlhorn admitted that alternative (ii) would have been obvious over alternative (i) [Reply to Restriction Requirement, Paper No. 13, p. 2, lines 5-9; Amendment and Renewed Request for Interference, Paper No. 16, pp. 3-4];
(b) adding: (i) to the thus-obtained acidic liposome-containing aqueous medium a charged chemical species which is cationic or	adding a catecholamine (epinephrine, norepinephrine or dopamine) to the external liposome phase [p. 270, lines 12-14] (to which sodium hydroxide was first added to create a transmembrane gradient of 3 pH units [p. 270, lines 10-12])

(ii) to the thus-obtained basic liposome-containing aqueous medium a charged chemical species which is anionic, and

Mehlhorn admitted that alternative (ii) would have been obvious over alternative (i) [Reply to Restriction Requirement, Paper No. 13, p. 2, lines 5-9; Amendment and Renewed Request for Interference, Paper No. 16, pp. 3-4];

(c) adding to the external liposome phase:

(i) a base to thereby induce the cationic chemical species to pass into the liposomes' internal acidic aqueous phase or

adding to the external liposome phase, before addition of the catecholamine, sodium hydroxide to establish a transmembrane gradient of 3 pH units, which induced the subsequently added catecholamine to accumulate inside the liposomes [p. 269, line 17 to p. 270, line 4; p. 270, lines 10-12];

(ii) an acid to thereby induce the anionic chemical species to pass into the liposomes' internal basic aqueous phase.

Mehlhorn admitted that alternative (ii) would have been obvious over alternative (i) [Reply to Restriction Requirement, Paper No. 13, p. 2, lines 5-9; Amendment and Renewed Request for Interference, Paper No. 16, pp. 3-4].

Nichols teaches performing the claimed steps in the sequence (a); (c); (b). In view of Cramer, it would have been obvious to perform the steps in the order (a); (b); (c). Cramer teaches preparing liposomes containing a charged chemical species by (a) forming liposomes in an aqueous medium containing a base; (b) adding a predominantly anionic chemical species to the external liposome phase; and (c) adding an acid to the external liposome phase to induce accumulation of the predominantly anionic chemical species inside the liposome [pp. 296-297, MATERIALS AND METHODS]; Cramer further teaches that loading liposomes using Nichols' sequence of steps occurs by "an analogous mechanism" to loading using Cramer's sequence of steps [p. 295, INTRODUCTION, line 11 to p. 296, line 2].

28. The method of Claim 27, wherein in (a)(i) an acidic liposome-containing aqueous medium is formed in which the acid is present in both the internal and external liposome phases, and in (c)(i) a base is added to the external phase to thereby induce the cationic chemical species to pass into the liposomes' internal aqueous phase.	Nichols teaches forming liposomes from egg PC in an aqueous citrate-phosphate buffer at pH 5 containing citric acid; the citric acid is present in both the internal and external liposome phases [p. 270, lines 5-10]; and adding sodium hydroxide to the external liposome phase to cause the subsequently added catecholamine to pass into the vesicles' internal aqueous phase [p. 270, lines 10-12]; Cramer teaches forming liposomes in an aqueous medium containing a base; and adding an acid to the external liposome phase to induce accumulation of the predominantly anionic chemical species inside the liposome [pp. 296-297, MATERIAL AND METHODS]; Cramer further teaches that loading liposomes using Nichols' sequence of steps occurs by "an analogous mechanism" to loading using Cramer's sequence of steps [p. 295, INTRODUCTION, line 11 to p. 296, line 2].
29. The method of Claim 27, wherein in (a)(ii) a basic liposome-containing aqueous medium is formed in which the base is present in both the internal and external liposome phases, and in (c) (ii) an acid is added to the external phase to thereby induce the anionic chemical species to pass into the liposomes' internal aqueous phase.	Mehlhorn admitted that alternative (ii) would have been obvious over alternative (i) [Reply to Restriction Requirement, Paper No. 13, p. 2, lines 5-9; Amendment and Renewed Request for Interference, Paper No. 16, pp. 3-4].
30. The method of Claim 27, wherein the aqueous medium containing the acid used in forming the liposomes in (a)(i), or the aqueous medium containing the base used in forming the liposomes in (a)(ii), is buffered.	Nichols teaches using a citrate-phosphate buffer containing citric acid as the aqueous medium for forming the liposomes [p. 270, lines 5-7].

31. The method of Claim 27, wherein the base which is added to thereby induce the cationic species to pass into the liposomes' internal aqueous phase in (c)(i), or the acid which is added to thereby induce the anionic chemical species to pass into the liposomes' internal aqueous phase in (c)(ii) is a component of a buffer.	Nichols teaches titrating with aqueous sodium hydroxide to establish a pH gradient and thereby induce accumulation of the catecholamine in the liposomes' internal aqueous phase [p. 270, lines 10-12]; the sodium hydroxide raises in situ the concentration of the basic components of the external citrate-phosphate buffer.
32. The method of Claim 30 wherein the base which is added to thereby induce the cationic species to pass into the liposomes' internal aqueous phase in (c)(i), or the acid which is added to thereby induce the anionic chemical species to pass into the liposomes' internal aqueous phase in (c)(ii), is a component of a buffer.	Nichols teaches titrating with aqueous sodium hydroxide to establish a pH gradient and thereby induce accumulation of the catecholamine in the liposomes' internal aqueous phase [p. 270, lines 10-12]; the sodium hydroxide raises in situ the concentration of the basic components of the external citrate-phosphate buffer.
33. The method of Claim 27, wherein the charged chemical species is a drug.	Nichols teaches a method of preparing liposomes containing catecholamines [p. 269, Summary, lines 2-4]; the catecholamines (dopamine, epinephrine or norepinephrine) are drugs, i.e., exhibit pharmacological activity (e.g., dopamine exhibits antitumor activity).
34. The method of Claim 27 wherein the charged chemical species is a hydrophobic drug.	Nichols teaches a method of preparing liposomes containing catecholamines [p. 269, Summary, lines 2-4]; the catecholamines (dopamine, epinephrine or norepinephrine) are drugs, i.e., exhibit pharmacological activity (e.g., dopamine exhibits antitumor activity); the catecholamines are relatively hydrophobic (lipophilic) as evidenced by their ability to pass through the lipid membrane.

35. The method of Claim 30 wherein the charged chemical species is a hydrophobic drug.

Nichols teaches a method of preparing liposomes containing catecholamines [p. 269, Summary, lines 2-4]; the catecholamines (dopamine, epinephrine or norepinephrine) are drugs, i.e., exhibit pharmacological activity (e.g., dopamine exhibits antitumor activity); the catecholamines are relatively hydrophobic (lipophilic) as evidenced by their ability to pass through the lipid membrane.

36. The method of Claim 31 wherein the charged chemical species is a hydrophobic drug.

Nichols teaches a method of preparing liposomes containing catecholamines [p. 269, Summary, lines 2-4]; the catecholamines (dopamine, epinephrine or norepinephrine) are drugs, i.e., exhibit pharmacological activity (e.g., dopamine exhibits antitumor activity); the catecholamines are relatively hydrophobic (lipophilic) as evidenced by their ability to pass through the lipid membrane.

37. The method of Claim 32 wherein the charged chemical species is a hydrophobic drug.

Nichols teaches a method of preparing liposomes containing catecholamines [p. 269, Summary, lines 2-4]; the catecholamines (dopamine, epinephrine or norepinephrine) are drugs, i.e., exhibit pharmacological activity (e.g., dopamine exhibits antitumor activity); the catecholamines are relatively hydrophobic (lipophilic) as evidenced by their ability to pass through the lipid membrane.

38. A method of preparing a liposome entrapped charged chemical species which comprises:

Nichols teaches a method of preparing liposomes containing catecholamines [p. 269, Summary, lines 2-4], by:

(a) forming liposomes in:

(i) an aqueous medium containing an acid

forming liposomes from egg phosphatidylcholine in an aqueous citrate-phosphate buffer containing citric acid [p. 270, lines 5-7]

which is substantially impermeable through the vesicle	which is "substantially impermeable" through the vesicle (as evidenced by establishment of a pH gradient upon addition of sodium hydroxide to the external liposome phase [p. 270, lines 10-12; March 22, 1993 Amendment, Paper No. 9, p. 10, lines 7-17])
to give an acidic liposome-containing aqueous medium in which the acid is present in the internal and external liposome phases; or	to give a citrate-phosphate buffer at pH 5 containing liposomes; citric acid is present in both the internal and external liposome phases [p. 270, lines 7-9];
(ii) an aqueous medium containing a base which is substantially impermeable through the vesicle to give a basic liposome-containing aqueous medium in which the base is present in the internal and external liposome phases;	Mehlhorn admitted that alternative (ii) would have been obvious over alternative (i) [Reply to restriction Requirement, Paper No. 13, p. 2, lines 5-9; Amendment and Renewed Request for Interference, Paper No. 16, pp. 3-4].
(b) adding: (i) to the thus-obtained acidic liposome-containing aqueous medium a charged chemical species which is cationic or	adding a catecholamine (epinephrine, norepinephrine or dopamine) to the external liposome phase [p. 270, lines 12-14] (to which sodium hydroxide was first added to create a transmembrane gradient of 3 pH units [p. 270, lines 10-12])
(ii) to the thus-obtained basic liposome-containing aqueous medium a charged chemical species which is anionic, and	Mehlhorn admitted that alternative (ii) would have been obvious over alternative (i) [Reply to Restriction Requirement, Paper No. 13, p. 2, lines 5-9; Amendment and Renewed Request for Interference, Paper No. 16, pp. 3-4];
(c) adding to the external liposome phase: (i) a base in an amount effective to create a pH gradient between the external liposome phase and the internal liposome phase to thereby induce the cationic chemical species to pass into the liposomes' internal acidic aqueous phase or	adding to the external liposome phase, before addition of the catecholamine, sodium hydroxide to establish a transmembrane gradient of 3 pH units, which induced the subsequently added catecholamine to accumulate inside the liposomes [p. 269, line 17 to p. 270, line 4; p. 270, lines 10-12];

(ii) an acid in an amount effective to create a pH gradient between the external liposome phase and the internal liposome phase to thereby induce the anionic chemical species to pass into the liposomes' internal basic aqueous phase.

Mehlhorn admitted that alternative (ii) would have been obvious over alternative (i) [Reply to Restriction Requirement, Paper No. 13, p. 2, lines 5-9; Amendment and Renewed Request for Interference, Paper No. 16, pp. 3-4].

Nichols teaches performing the claimed steps in the sequence (a); (c); (b). In view of Cramer, it would have been obvious to perform the steps in the order (a); (b); (c). Cramer teaches preparing liposomes containing a charged chemical species by (a) forming liposomes in an aqueous medium containing a base; (b) adding a predominantly anionic chemical species to the external liposome phase; and (c) adding an acid to the external liposome phase to induce accumulation of the anionic chemical species inside the liposome [pp. 296-297, MATERIALS AND METHODS]; Cramer further teaches that loading liposomes using Nichols' sequence of steps occurs by "an analogous mechanism" to loading using Cramer's sequence of steps [p. 295, INTRODUCTION, line 11 to p. 296, line 2].

39. The method of Claim 38, wherein in (a) (i) an acidic liposome-containing aqueous medium is formed in which the acid is present in both the internal and external liposome phases, and in (c) (i) a base is added to the external phase to thereby induce the cationic chemical species to pass into the liposomes' internal aqueous phase.

Nichols teaches forming liposomes from egg PC in an aqueous citrate-phosphate buffer at pH 5 containing citric acid; the citric acid is present in both the internal and external liposome phases [p. 270, lines 5-10]; and adding sodium hydroxide to the external liposome phase to cause the subsequently added catecholamine to pass into the vesicles' internal aqueous phase [p. 270, lines 10-12);

	Cramer teaches forming liposomes in an aqueous medium containing a base; and adding an acid to the external liposome phase to induce accumulation of the predominantly anionic chemical species inside the liposome [pp. 296-297, MATERIALS AND METHODS]; Cramer further teaches that loading liposomes using Nichols' sequence of steps occurs by "an analogous mechanism" to loading using Cramer's sequence of steps [p. 295, INTRODUCTION, line 11 to p. 296, line 2].
40. The method of Claim 38, wherein in (a) (ii) a basic liposome-containing aqueous medium is formed in which the base is present in both the internal and external liposome phases, and in (c) (ii) an acid is added to the external phase to thereby induce the anionic chemical species to pass into the liposomes' internal aqueous phase.	Mehlhorn admitted that alternative (ii) would have been obvious over alternative (i) [Reply to Restriction Requirement, Paper No. 13, p. 2, lines 5-9; Amendment and Renewed Request for Interference, Paper No. 16, pp. 2-4].
41. The method of Claim 38 wherein the aqueous medium containing the acid used in forming the liposomes in (a) (i), or the aqueous medium containing the base used in forming the liposomes in (a) (ii), is buffered.	Nichols teaches using a citrate-phosphate buffer containing citric acid as the aqueous medium for forming the liposomes [p. 270, lines 5-7].
42. The method of Claim 38, wherein the base which is added to thereby induce the cationic chemical species to pass into the liposomes' internal aqueous phase in (c) (i), or the acid which is added to thereby induce the anionic chemical species to pass into the liposomes' internal aqueous phase in (c) (ii), is a component of a buffer.	Nichols teaches titrating with aqueous sodium hydroxide to establish a pH gradient and thereby induce accumulation of the catecholamine in the liposomes' internal aqueous phase [p. 270, lines 10-12]; the sodium hydroxide raises <u>in situ</u> the concentration of the basic components of the external citrate-phosphate buffer.

43. The method of Claim 41 wherein the base which is added to thereby induce the cationic chemical species to pass into the liposomes' internal aqueous phase in (c) (i), or the acid which is added to thereby induce the anionic chemical species to pass into the liposomes' internal aqueous phase in (c) (ii), is a component of a buffer.	Nichols teaches titrating with aqueous sodium hydroxide to establish a pH gradient and thereby induce accumulation of the catecholamine in the liposomes' internal aqueous phase [p. 270, lines 10-12]; the sodium hydroxide raises in situ the concentration of the basic components of the external citrate-phosphate buffer.
44. The method of Claim 38 wherein the charged chemical species is a drug.	Nichols teaches a method of preparing liposomes containing catecholamines [p. 269, Summary, lines 2-4]; the catecholamines (dopamine, epinephrine or norepinephrine) are drugs, i.e., exhibit pharmacological activity (e.g., dopamine exhibits antitumor activity).
45. The method of Claim 38 wherein the charged chemical species is a hydrophobic drug.	Nichols teaches a method of preparing liposomes containing catecholamines [p. 269, Summary, lines 2-4]; the catecholamines (dopamine, epinephrine or norepinephrine) are drugs, i.e., exhibit pharmacological activity (e.g., dopamine exhibits antitumor activity); the catecholamines are relatively hydrophobic (lipophilic) as evidenced by their ability to pass through the lipid membrane.
46. The method of Claim 41 wherein the charged chemical species is a hydrophobic drug.	Nichols teaches a method of preparing liposomes containing catecholamines [p. 269, Summary, lines 2-4]; the catecholamines (dopamine, epinephrine or norepinephrine) are drugs, i.e., exhibit pharmacological activity (e.g., dopamine exhibits antitumor activity); the catecholamines are relatively hydrophobic (lipophilic) as evidenced by their ability to pass through the lipid membrane.

47. The method of Claim 42 wherein the charged chemical species is a hydrophobic drug.	Nichols teaches a method of preparing liposomes containing catecholamines [p. 269, Summary, lines 2-4]; the catecholamines (dopamine, epinephrine or norepinephrine) are drugs, i.e., exhibit pharmacological activity (e.g., dopamine exhibits antitumor activity); the catecholamines are relatively hydrophobic (lipophilic) as evidenced by their ability to pass through the lipid membrane.
48. The method of Claim 43 wherein the charged chemical species is a hydrophobic drug.	Nichols teaches a method of preparing liposomes containing catecholamines [p. 269, summary, lines 2-4]; the catecholamines (dopamine, epinephrine or norepinephrine) are drugs, i.e., exhibit pharmacological activity (e.g., dopamine exhibits antitumor activity); the catecholamines are relatively hydrophobic (lipophilic) as evidenced by their ability to pass through the lipid membrane.
49. The method of Claim 38 wherein said aqueous medium containing an acid in step (a) (i) has a pH less than 7 and wherein said aqueous medium containing a base in step (a) (ii) has a pH greater than 7.	Nichols teaches forming liposomes in an aqueous medium containing citric acid which has a pH of 5 [p. 270, lines 5-7].
50. The method of Claim 49 wherein said aqueous medium containing an acid in step (a) (i) has a pH of 5.0 and wherein said base added to the external liposome phase in step (c) (i) raises the pH of the external liposome phase to 7.4.	Nichols teaches forming liposomes in an aqueous medium containing citric acid which has a pH of 5 [p. 270, lines 5-7] and adding sufficient aqueous sodium hydroxide to the external liposome phase to raise the pH of the external phase to 8 [p. 270, lines 10-12]. There is no qualitative difference in the choice of a pH of 8 versus 7.4 in this application. Mehlhorn admitted that the particular pH of the aqueous medium was immaterial so long as a pH gradient was established [March 22, 1993 Amendment, Paper No. 9, p. 6.]